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Hepatocyte Nuclear Factor 3/fork head Homolog 11 Is Expressed in Proliferating Epithelial and Mesenchymal Cells of Embryonic and Adult Tissues

HONGGANG YE,[1] THOMAS F. KELLY,[1] UZMA SAMADANI,[1] LORENA LIM,[1] SANDRINE RUBIO,[1] DAVID G. OVERDIER,[1]† KENNETH A. ROEBUCK,[2] AND ROBERT H. COSTA[1]*

Department of Biochemistry, University of Illinois at Chicago, Chicago, Illinois 60612-7334,[1] and Department of Immunology and Microbiology, Rush Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612[2]

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The hepatocyte nuclear factor 3α (HNF-3α) and 3β proteins have homology in the winged helix/fork head DNA binding domain and regulate cell-specific transcription in hepatocytes and in respiratory and intestinal epithelia. In this study, we describe two novel isoforms of the winged helix transcription factor family, HNF-3/fork head homolog 11A (HFH-11A) and HFH-11B, isolated from the human colon carcinoma HT-29 cell line. We show that these isoforms arise via differential splicing and are expressed in a number of epithelial cell lines derived from tumors (HT-29, Caco-2, HepG2, HeLa, A549, and H441). We demonstrate that differentiation of Caco-2 cells toward the enterocyte lineage results in decreased HFH-11 expression and reciprocal increases in HNF-3α and HNF-3β mRNA levels. In situ hybridization of 16 day postcoitus mouse embryos demonstrates that HFH-11 expression is found in the mesenchymal and epithelial cells of the liver, lung, intestine, renal cortex, and urinary tract. Although HFH-11 exhibits a wide cellular expression pattern in the embryo, its adult expression pattern is restricted to epithelial cells of Lieberkühn's crypts of the intestine, the spermatocytes and spermatids of the testis, and the thymus and colon. HFH-11 expression is absent in adult hepatocytes, but its expression is reactivated in proliferating hepatocytes at 4, 24, and 48 h after partial hepatectomy. Consistent with these findings, we demonstrate that HFH-11 mRNA levels are stimulated by intratracheal administration of keratinocyte growth factor in adult lung and its expression in an adult endothelial cell line is reactivated in response to oxidative stress. These experiments show that the HFH-11 transcription factor is expressed in embryonic mesenchymal and epithelial cells and its expression is reactivated in these adult cell types by proliferative signals or oxidative stress.

Cell-specific transcription relies on the combinatorial recognition of multiple *cis*-acting elements by families of cell-restricted transcription factors (80). One of these regulatory families is represented by the hepatocyte nuclear factor 3α (HNF-3α), HNF-3β, and HNF-3γ proteins (43), which have homology in the winged helix DNA binding domain (12) and function in combination with other liver-enriched transcription factors to mediate hepatocyte-enriched transcription (17). The HNF-3α and -3β proteins also activate the transcription of genes important for respiratory epithelial cell function (7, 14, 35, 40, 60, 82). The HNF-3 proteins thus appear to play an important transcriptional regulatory role in epithelial cell type-specific gene expression in adult tissues derived from gut endoderm.

In the adult intestine, multipotent proliferative stem cells in Lieberkühn's crypts in the mouse intestine give rise to four terminally differentiated cell types: digestive and absorptive columnar enterocytes (representing the most abundant cell type), mucus-producing goblet cells, enteroendocrine cells, and Paneth cells (53). As the postmitotic enterocytes, goblet cells, and enteroendocrine cells exit Lieberkühn's crypts, they terminally differentiate during their migration toward the tip of the intestinal villus and function to replenish the short-lived differentiated epithelium of the villus (53). By contrast, differentiation of the Paneth cells occurs as they descend to the base of the crypt and function in the defense against pathogenic organisms (8). Terminal differentiation is accompanied by the induction of cell-specific marker genes which vary in their expression pattern along the crypt-to-villus and duodenum-to-ileum axes (69). The POU homeodomain HNF-1 (61), orphan steroid hormone receptor HNF-4 (23, 65), bZIP C/EBPα (10), and homeodomain cdx-2 (37) transcription factors regulate intestinal epithelium gene expression (5, 16, 28, 46, 63, 77) and exhibit distinct cellular expression patterns in the adult intestinal epithelium (10, 37, 61, 65). Although the HNF-3α and -3β genes are expressed in the epithelia of embryonic and adult intestines (38, 47), the roles of HNF-3 and winged helix transcription factors in adult intestinal epithelial cell differentiation have not been studied.

The HNF-3 and *Drosophila* homeotic fork head (fkh) (72) proteins are the first two members of an extensive family of cell-specific transcription factors which have homology in the winged helix domain and participate in the differentiation of diverse cellular lineages (for reviews, see references 17 and 34). Targeted gene disruptions in mice demonstrate that the winged helix transcription factors are involved in embryonic pattern formation. The HNF-3α and HNF-3β genes are expressed during the primitive streak stage of embryogenesis (3, 47, 58, 59). Disruption of the HNF-3β gene in homozygous knockout mice causes an embryonic lethal phenotype, exhibiting defects in the formation of notochord, neurotube, somites, and gut endoderm (2, 73). The winged helix transcrip-

* Corresponding author. Mailing address: Department of Biochemistry (M/C 536), University of Illinois at Chicago, College of Medicine, 1819 W. Polk St., Chicago, IL 60612-7334. Phone: (312) 996-0474. Fax: (312) 413-0364. E-mail: RobCosta@uic.edu.
† Present address: Panduit Corp., Tinley Park, IL 60477.

tion factor brain factor 1 (BF-1) is involved in morphogenesis of the telencephalon, and mice homozygous for a BF-1 null mutant genotype exhibit dramatic reductions in the size of their cerebral hemispheres and die at birth (79). Targeted disruption of the BF-2 gene in mice results in inhibited induction of renal mesenchyme into tubular epithelium and branching of the ureter and renal collecting system (32). Furthermore, a recent study has demonstrated that disruption of the winged helix *whn* gene is responsible for the nude mouse phenotype (49). Taken together, these embryonic studies indicate that the winged helix transcription factor family plays an integral regulatory role in cellular commitment events.

In this study we report on the isolation of two isoforms of the winged helix transcription factor family, HFH-11A and HFH-11B, which are related by differential splicing and are expressed in a number of epithelial cell lines derived from tumors. We show that expression of the HFH-11 isoforms decreases following differentiation of human colon carcinoma Caco-2 cells toward the enterocyte cell lineage, whereas HNF-3 expression exhibits a reciprocal increase in response to differentiation. In situ hybridization analysis enabled us to determine that HFH-11 is expressed in proliferating mesenchymal and epithelial cells of the embryonic liver, lung, intestine, and renal cortex. We present in vivo experiments to indicate that the HFH-11 transcription factor is induced by proliferative signals in adult epithelial cells. We also show that HFH-11 expression is induced in response to oxidative stress in an adult endothelial cell line.

MATERIALS AND METHODS

Construction of HFH-11 cDNA chimeras, cotransfection assays, and analysis of protein production via Western blot analysis. In order to determine the HFH-11 transcriptional activity, cotransfection assays were performed in human colon carcinoma Caco-2 cell lines with a reporter plasmid containing five copies of the HFH-11 #24 binding site (a DNA binding site selected in vitro by HFH-11 recombinant protein; see below) upstream of a TATA box-driven luciferase gene (pGL2 basic vector; Promega) and an expression vector that used the cytomegalovirus (CMV) promoter to express the HFH-11 cDNA sequences. A CMV promoter-driven β-galactosidase control plasmid was used to normalize for differences in transfection efficiency (51). The full-length HFH-11A or HFH-11B cDNA was cloned as an *Eco*RI-*Xba*I fragment (5′→3′) in the CMV expression vector (51). A common *Kpn*I restriction cleavage site in the cDNAs was located between exon A1 and exon A2 at amino acids 379 and 364 of the HFH-11A and HFH-11B isoforms, respectively. We used the *Kpn*I site to create chimeric proteins between the HFH-11 isoforms which allowed us to examine the transcriptional activity of the C-terminal region.

Human colon carcinoma Caco-2 cells were maintained in monolayer cultures and grown in Ham's F12/Dulbecco modified Eagle medium supplemented with 20% heat-inactivated fetal bovine serum, 100 U of penicillin, and 100 μg of streptomycin per ml (all from GIBCO/BRL Laboratories). Cells were transfected (55) with Lipofectin reagent (GIBCO/BRL) according to the manufacturer's protocol (35-mm-diameter plates, 400 ng of CMV–HFH-11 expression vector, 1,600 ng of reporter plasmid containing five copies of the HFH-11 #24 binding site upstream of the luciferase gene 100 ng of CMV–β-galactosidase construct, and 10 μl of Lipofectin). Cellular protein extracts were prepared from transfected cells 48 h after transfection and analyzed for luciferase enzyme activity with a commercially available luciferase assay system (Promega). β-Galactosidase enzyme activity was determined as described previously (54, 55). For the generation of an HFH-11-specific antibody, HFH-11 N-terminal sequences (amino acids 1 to 138) were fused to glutathione *S*-transferase (GST) and recombinant HFH-11 protein was isolated from bacterial cultures, purified to homogeneity via glutathione affinity chromatography (50), and used to immunize rabbits (antisera were raised by the University of Illinois at Chicago animal facility). The primers used to PCR amplify the HFH-11 N-terminal sequences and allow its cloning in frame with the GST protein are the following: 5′-gcg ggatcc ata atg aaa act agc ccc and 5′-gcg tctaga cag ggt cac ttc tgt cct. Affinity purification of HFH-11 antisera was performed as described previously (36). To determine the expression of HFH-11A, HFH-11B, and chimeric protein during cotransfection experiments, nuclear extracts were prepared from Caco-2 cells transfected with the HFH-11 cDNA constructs and analyzed by Western blotting with HFH-11 antibody by using protocols described previously (54). Nuclear extracts were prepared from nontransfected subconfluent human colon carcinoma HT-29 and Caco-2 cell lines, and HFH-11 protein expression was measured via Western blot analysis.

In situ hybridization of stage-specific embryos and RNase protection assays. In situ hybridization of paraffin-embedded mouse embryos was performed with ^{33}P-labeled antisense HFH-11 RNA probes generated from the two rat HFH-11 cDNA *Eco*RI-*Pst*I pGEM1 subclones (450 bp) by using the appropriate RNA polymerase enzyme and procedures described by Duncan et al. (23). For the HFH-4 in situ hybridization with adult mouse testis, an *Xba*I-linearized HFH-4 full-length cDNA pGEM1 (*Xba*I-*Sal*I) template and SP6 RNA polymerase were used (14). Antisense ^{33}P-labeled RNA probes were hybridized to sectioned dewaxed tissue, rinsed at high stringency, and subjected to autoradiography (23). A dark-field condenser was used to enhance the visualization of the silver grains due to specific HFH-11 hybridization.

RNase protection assays were performed as described previously (18, 54, 55). The HFH-11 riboprobe was synthesized by using T7 RNA polymerase from a *Hin*dIII-digested HFH-11 rat cDNA template (450-bp *Eco*RI-*Pst*I HFH-11 winged helix fragment subcloned in pGEM1). For RNase protection with human colon carcinoma HT-29 and Caco-2 cell lines, we generated an antisense probe from the HFH-11A cDNA which contained exon A2 sequences and 58 amino acids N terminal to exon A2 (amino acids 366 to 469). The probe was made via PCR with the following primers: 5′-ctc ggatcc aag atg aag cca ctg cta and 5′-ctc gaattc agc tat ccc ctc ctc agc. The 409-nucleotide *Bam*HI-*Eco*RI HFH-11A fragment was subcloned in pGEM1, and T7 polymerase was used to generate antisense RNA probe from the *Bam*HI-digested template.

In vitro DNA binding site selection and EMSA. The primers used to PCR amplify the HFH-11 winged helix DNA binding domain and clone into the GST expression vector are the following: 5′-tct ggatcc aag gag aat tgt cac ctg and 5′-ctc tctaga gag ttc ggt ttt gat ggt (HFH-11A amino acids 210 to 359; HFH-11B amino acids 210 to 344). The affinity-purified GST–HFH-11 fusion protein was used to isolate high-affinity HFH-11 binding sites from a pool of partially degenerate oligonucleotides containing 18 degenerate positions by a process of repetitive protein selection and PCR amplification as described previously in our laboratory (50) and known as the sequential selection and amplification of binding sites (SAAB) protocol (6). The HFH-11 protein-selected SAAB sites were cloned in pGEM1, the DNA insert was labeled during PCR amplification by using 5′-labeled T7 and SP6 primers and tested for HFH-11 protein complex formation by electrophoretic mobility shift assays (EMSA) with 500 ng of recombinant GST–HFH-11 fusion protein by methods described previously (50). We searched 27 promoter regions of genes expressed in the intestinal epithelium, and 11 of these promoters contained putative HNF-3 binding sites. These 11 promoter regions were then screened for homology with HFH-11 binding sequences, and five promoter regions were selected for EMSA. Double-stranded oligonucleotides were made to the HFH-11 24 site and several HFH-11 and HNF-3 potential binding sites in promoters of genes expressed in intestinal epithelial cells. These sites were used for EMSA with 500 ng of GST–HFH-11 fusion protein or in vitro-translated HNF-3 proteins as described previously (50). A 200-fold molar excess of unlabeled double-stranded oligonucleotide was added to the binding reaction mixtures for the competition lanes.

Intratracheal administration of KGF and hydrogen peroxide treatment of endothelial cells. Intratracheal administration of keratinocyte growth factor (KGF) was performed as described by Ulich et al. (71). Male Lewis rats (200 to 250 g; 12 to 16 weeks old) were anesthetized with ether and prepped for surgery via shaving and Betadine scrub. An incision through the skin and subcutaneous tissues overlying the trachea was made, and the strap muscles were separated away from the trachea with blunt forceps. KGF (generous gift of Amgen, courtesy of Thomas Ulich) (5 mg/kg of body weight) resuspended in sterile phosphate-buffered saline (PBS) was intratracheally administered with a 25-gauge needle. Control animals were injected with PBS alone. The incision was closed with intermittent sutures. The rats were restored to consciousness by normal ventilation and then sacrificed by CO_2 asphyxiation and guillotine for harvesting of the entire lungs at time points 6, 12, 18, 24, 72, and 240 h after KGF administration. RNA was extracted from the lungs via polytron homogenization with the RNAzol reagent (Tel-Test) according to manufacturer protocol.

Human microvessel endothelial cells (HMEC) were serum starved for 24 h and then treated with increasing concentrations of hydrogen peroxide for 1 h prior to harvesting total RNA with RNAzol reagent. RNA expression levels were analyzed by Northern blot analysis by using radioactively labeled HFH-11 or HFH-8 cDNA probe.

Liver regeneration rat protocol. Liver regeneration experiments were done as described previously (55). Male Fisher rats (approximately 200 g) were anesthetized with ether and subjected to midventral laparotomy with approximately 70% liver resection (left lateral and median lobes). Control sham operations were performed by subjecting rats to midventral laparotomy, gentle handling of the liver, and then closure with intermittent sutures. The animals were restored to consciousness by normal ventilation and then sacrificed by CO_2 asphyxiation and guillotine 1, 2, 4, 24, 48, and 72 h after operation for harvesting of the entire liver. Two-thirds of the harvested organ was used to generate RNA with the RNAzol reagent, while the remaining one-third was flash-frozen on dry ice and stored at −70°C for in situ hybridization. Livers from untreated animals were used as controls.

Nucleotide sequence accession numbers. The sequences for the HFH-11A and HFH-11B cDNAs have been submitted to GenBank, and the GenBank accession numbers are U74612 and U74613, respectively.

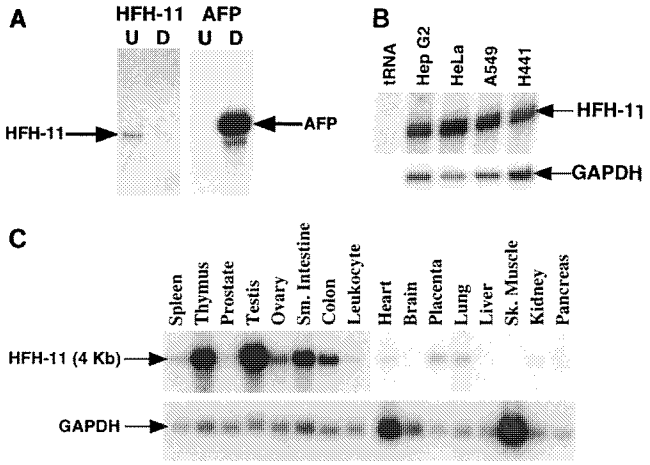


FIG. 1. HFH-11 expression in cell lines and adult tissues. (A) HFH-11 expression decreases during Caco-2 cell differentiation. RNA was isolated from undifferentiated (U) (90% confluent) or differentiated (D) (14 days of confluent cell density) Caco-2 cells and subjected to Northern blot analysis (15) with the HFH-11 cDNA hybridization probe. The identical blot was then stripped and probed with the α-fetoprotein (AFP) cDNA probe, which is induced during Caco-2 differentiation (70). (B) HFH-11 is expressed in a number of epithelial cell lines. Total RNA was isolated from human hepatoma HepG2, cervical carcinoma HeLa, and pulmonary adenocarcinoma A549 and H441 cells and then analyzed for HFH-11 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression via RNase protection assay (see Materials and Methods). Note that the HFH-11B isoform is the RNase-protected fragment shown and is the most prominent isoform expressed in these epithelial cell lines. We also included hybridization with tRNA as a control for nonspecific bands (tRNA). (C) Expression of HFH-11 in adult human organs via Northern blot analysis. Northern blots were hybridized with HFH-11 and GAPDH cDNA probes as described by the manufacturer (Clontech). Northern blots demonstrate that HFH-11 is abundantly expressed in thymus, testis, small intestine (Sm. Intestine), and colon. Sk. Muscle, skeletal muscle.

RESULTS

Isolation of a new winged helix transcription factor gene from intestinal HT-29 and Caco-2 cells. The human colon carcinoma Caco-2 cell line mimics a subset of the differentiation pathways that occur from the crypt-to-villus axis of the adult intestine (75). Caco-2 cells remain undifferentiated during low-density passages, but after several days at confluent density, cells begin to differentiate toward the enterocyte lineage and acquire expression of enterocyte-specific marker genes (11). In order to isolate novel winged helix transcription factors important for intestinal differentiation, we used cDNA from Caco-2 cells for PCR amplification with degenerate primers synthesized to conserved amino acid regions of the winged helix DNA binding domain as described previously (15). These PCR products were cloned, sequenced, and analyzed by Northern blot analysis for changes in expression during Caco-2 differentiation. One of these winged helix family members, HNF-3/fork head homolog 11 (HFH-11), exhibited decreased expression following Caco-2 cell differentiation (Fig. 1A, compare lanes U and D). Expression of the α-fetoprotein gene which is induced during Caco-2 cell differentiation (70) was used to monitor Caco-2 differentiation (Fig. 1A, lane D). This winged helix gene was also expressed in a number of established epithelial cell lines, including human hepatoma HepG2, cervical carcinoma HeLa, and pulmonary adenocarcinoma cell lines A549 and H441 (Fig. 1B). Northern blot analysis with adult tissue RNA (Fig. 1C) demonstrates that HFH-11 is not only expressed in small intestine and colon but also displays high mRNA levels in the thymus and testis. Moderate HFH-11 expression was found in the ovary, and substantially reduced mRNA levels were found in the spleen, leukocytes, heart, placenta, lung, and kidney.

In order to isolate a full-length cDNA clone, the HFH-11 clone was used to screen a cDNA library prepared from undifferentiated human intestinal HT-29 cells (75). Thirteen positive cDNA clones were isolated, several of which contained 3.8-kb cDNA inserts. The sizes of these HFH-11 cDNA suggested that they were full-length clones based on the size of HFH-11 mRNA from Northern blot analysis (Fig. 1C). After further characterization, we found that the HFH-11 cDNAs were composed of at least two differentially spliced isoforms (denoted as A and B). The HFH-11A (Fig. 2A) and HFH-11B cDNAs encode 801- and 748-amino-acid polypeptides, respectively, and possess a winged helix domain exhibiting 39% amino acid identity compared to that of the HNF-3 protein. Except for two additional HFH-11A exons, the HFH-11 cDNA isoforms have identical nucleotide sequences. Exon A1 encodes a 15-amino-acid insertion in the wing 2 region of the winged helix domain adjacent to the basic amino acid residues involved in a base-specific contact with the minor groove (12); exon A2 encodes a 38-amino-acid insertion at the C terminus (Fig. 2A). The HFH-11 proteins contain two regions of PEST sequences (Fig. 2) which mediate rapid protein degradation of enzymes, transcription factors, and components of receptor signalling pathways (56). The PEST regions are rich in proline (P), glutamate (E), serine (S), and threonine (T) amino acids, range in length from 12 to 60 residues, and are often flanked by charged amino acid residues (56). Furthermore, several putative mitogen-activated protein (MAP) kinase phosphorylation sites (proline-dependent/p34^{cdc2}) (20, 48) are present in the C termini of the HFH-11 proteins, and a putative MAP2 phosphorylation site (68) is found in the HFH-11A exon A1 sequences of the winged helix DNA binding domain (Fig. 2A).

To demonstrate that HFH-11 protein is expressed in Caco-2 and HT-29 cells, nuclear extracts were prepared from undifferentiated cells and then analyzed for HFH-11 protein by Western blot analysis using affinity-purified antibody generated against the HFH-11 N-terminal sequences (see Materials and Methods). These data demonstrate that both Caco-2 and HT-29 human carcinoma cell lines expressed a 90-kDa HFH-11 protein which is consistent with the predicted molecular masses of both HFH-11 proteins (Fig. 2B). The HFH-11 antibody also detects immunoreactive protein migrating at larger molecular masses than the major band which may correspond to phosphorylated HFH-11 protein (Fig. 2B). A lower-molecular-mass product was also detected in the HT-29 nuclear extract, which may be due to proteolytic degradation.

In order to determine the relationship of the genomic exons with respect to splicing of the HFH-11 isoforms, human HFH-11 genomic clones were isolated and subjected to PCR analysis with specific HFH-11 primers spanning exon A1 and A2 sequences (data not shown). These experiments are summarized in Fig. 3, which depicts the locations of the HFH-11 A1 and A2 exons in the genomic clone with respect to the exons utilized in both HFH-11 isoforms (exon A/B). These data demonstrated that exons unique to HFH-11A are situated between exons shared by both mRNAs and are spliced into mature HFH-11A mRNA, whereas maturation of the HFH-11B mRNA involves deleting exon A1 and A2 sequences during RNA splicing.

Exon A2 sequences in the C terminus of HFH-11A isoform suppresses transcriptional activation. In order to obtain a DNA site which is recognized by the HFH-11 proteins, we used recombinant HFH-11A and HFH-11B for the SAAB protocol (6) as described previously (50). The HFH-11 protein-selected SAAB sites were cloned, and the DNA inserts were labeled

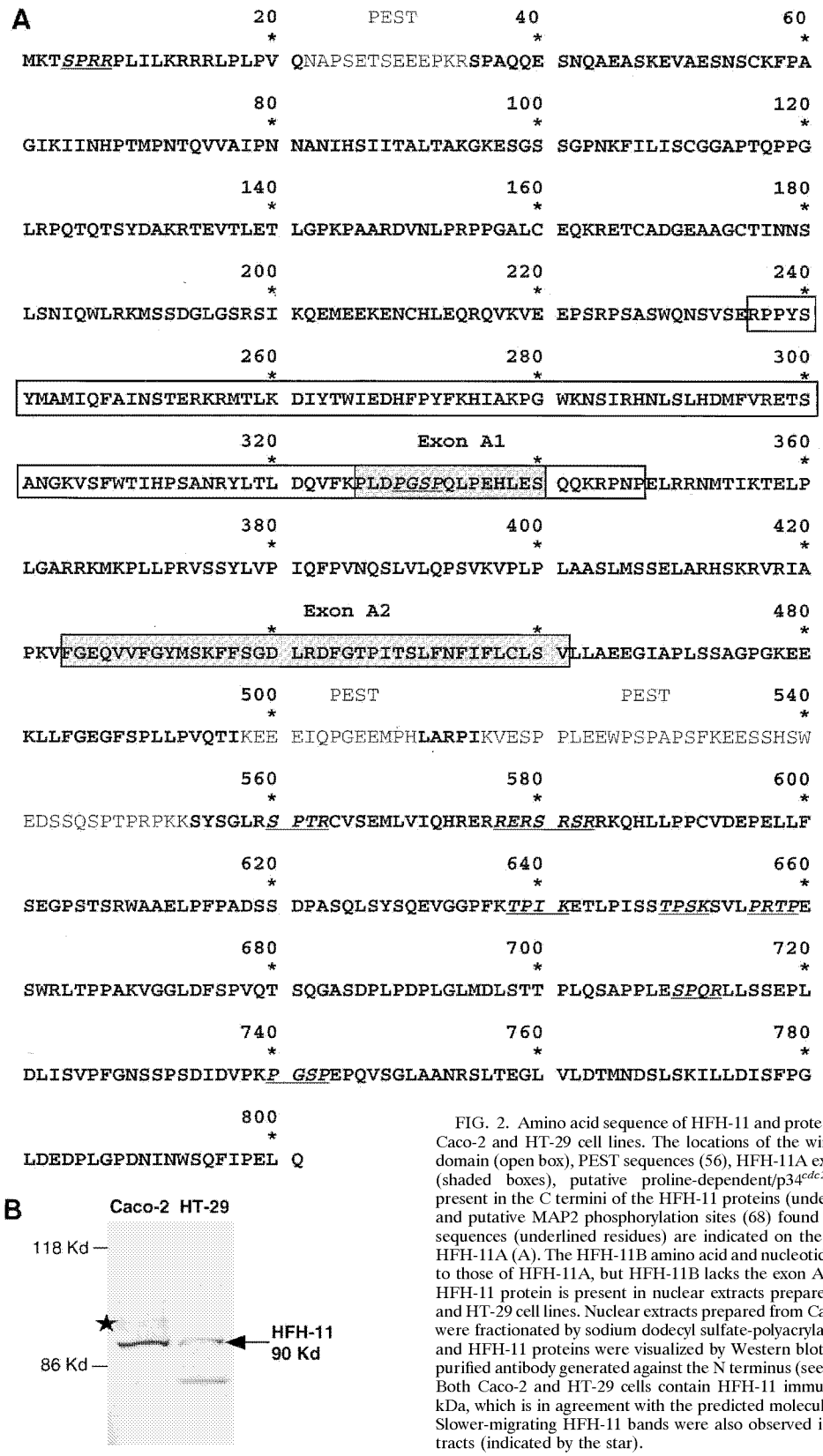


FIG. 2. Amino acid sequence of HFH-11 and protein expression in intestinal Caco-2 and HT-29 cell lines. The locations of the winged helix DNA binding domain (open box), PEST sequences (56), HFH-11A exon A1 and A2 sequences (shaded boxes), putative proline-dependent/p34^{cdc2} phosphorylation sites present in the C termini of the HFH-11 proteins (underlined residues) (20, 48), and putative MAP2 phosphorylation sites (68) found in the HFH-11 exon A1 sequences (underlined residues) are indicated on the amino acid sequence of HFH-11A (A). The HFH-11B amino acid and nucleotide sequences are identical to those of HFH-11A, but HFH-11B lacks the exon A1 and A2 sequences. (B) HFH-11 protein is present in nuclear extracts prepared from intestinal Caco-2 and HT-29 cell lines. Nuclear extracts prepared from Caco-2 and HT-29 cell lines were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and HFH-11 proteins were visualized by Western blot analysis with an affinity-purified antibody generated against the N terminus (see Materials and Methods). Both Caco-2 and HT-29 cells contain HFH-11 immunoreactive protein of 90 kDa, which is in agreement with the predicted molecular mass from the cDNA. Slower-migrating HFH-11 bands were also observed in the Caco-2 nuclear extracts (indicated by the star).

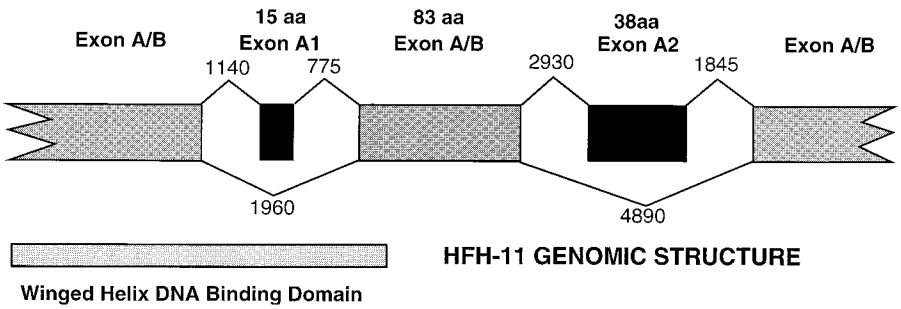


FIG. 3. Genomic organization of the HFH-11 gene. Primers specific to each of the exons surrounding the exon A1 and A2 sequences were used for PCR amplification and were cloned and sequenced to determine the position of the intron/exon boundary (data not shown). The results for the positions of the exon A1 and A2 sequences in the HFH-11 human genomic clone are shown schematically. Also indicated are the positions of the exon A/B sequences which are utilized by both isoforms and the approximate size of the introns in nucleotides, as determined by the sizes of the PCR products from the HFH-11 genomic clone. aa, amino acids.

and tested for HFH-11 protein-DNA complex formation by EMSA. One of these selected sites, HFH-11 #24 (5′-TACGT TGTTATTTGTTTTTTTCG), forms specific protein-DNA complexes with recombinant in vitro-translated HFH-11A and HFH-11B proteins which are not competed by HNF-3 binding sites (Fig. 4A, HFH-1 #3 and HNF-3). We next wanted to determine whether the HFH-11 proteins functioned as transcriptional activators or repressors. We used cotransfection assays in Caco-2 cells (51, 54) which consisted of a reporter plasmid containing five copies of the HFH-11 24 binding site upstream of a TATA box-driven luciferase gene and an expression vector that used the CMV promoter to express the HFH-11 cDNA sequences (51). A CMV promoter-driven β-galactosidase construct was included in the cotransfection assays as an internal control to normalize for differences in transfection efficiency (54, 55). Cotransfection assays with the HFH-11B cDNA expression vector provided an approximately 15-fold increase in reporter gene transcription over that with the CMV control plasmid (Fig. 4B, construct 2), whereas no transcriptional activation was observed with the HFH-11A expression construct (Fig. 4B, construct 1). Equivalent amounts of HFH-11 proteins were expressed in the transfection assays as determined by Western blot analysis (data not shown), and transcriptional activation required the presence of the HFH-11 #24 binding site. Because the HFH-11A and -B proteins are identical except for two exons, we used a *Kpn*I site which lies between these two exon sequences to create chimeras between the HFH-11A and -B proteins. Chimeric proteins containing the HFH-11A DNA binding domain fused to the HFH-11B C terminus exhibited transcriptional activation levels comparable to that of HFH-11B (Fig. 4B, constructs 2 and 3). In contrast, no transcriptional activation was observed with the chimera containing the HFH-11A C terminus fused to the HFH-11B DNA binding domain or with HFH-11 proteins lacking the C terminus (Fig. 4B, constructs 4 to 6). These transfection studies suggest that the HFH-11B C-terminal sequences are required for transcriptional activation and addition of the C-terminal exon A2 sequences in the HFH-11A protein suppresses this activity.

Differential expression of the winged helix transcription factor family during Caco-2 cellular differentiation. To further examine the expression pattern of HFH-11 during Caco-2 cell differentiation, we isolated RNA from undifferentiated Caco-2 cells (proliferating cells; 0 days) or differentiated Caco-2 cells (2, 11, or 17 days of confluent cell density) and examined HFH-11 expression via RNase protection assay. The human HFH-11 RNA probe contained sequences spanning exon A2 and generated RNase-protected fragments of different sizes for each of the HFH-11 isoforms. For comparison, we also examined the expression patterns of the well-characterized winged helix transcription factors HNF-3α and HNF-3β during Caco-2 cell differentiation. These RNase protection studies showed that the HFH-11 isoforms are expressed in undifferentiated Caco-2 cells and that the level of the transcriptional activator HFH-11B mRNA was higher than that for the transcriptionally inactive HFH-11A isoform (Fig. 5A, 0 days). HFH-11 is abundantly expressed during the initiation of cellular differentiation (Fig. 5A, 2 days) and begins to decline after 5 days of continuous confluent cell density (data not shown). A significant reduction in HFH-11 mRNA expression levels is found in fully differentiated Caco-2 cells (Fig. 5, 11 and 17 days). By contrast, HNF-3α expression slightly increases following Caco-2 differentiation, whereas the homeodomain cdx-2 and HNF-3β demonstrate a strong increase in expression when Caco-2 cells are maintained at confluent density for 2 days (Fig. 5, compare 0 and 2 days). This coincides with the initiation of Caco-2 cell differentiation, as demonstrated by the induction of apolipoprotein A1 gene expression (Fig. 5A), a known target gene for HNF-3β in the intestine (31, 57). These experiments indicated that following Caco-2 differentiation toward the enterocyte cell lineage, HFH-11 expression decreases and the HNF-3 mRNA level increases.

HFH-11 is expressed in proliferating epithelial and mesenchymal cells of the mouse embryo. To determine the cellular expression pattern of HFH-11 in mouse embryos, we used ^{33}P-labeled antisense rat HFH-11 RNA probe for in situ hybridization with sagittal sections of paraffin-embedded 16 day postcoitus (p.c.) mouse embryos (Fig. 6A). After hybridization, stringent washes, and autoradiography, dark-field microscopy (Fig. 6B) was used to visualize HFH-11-expressing cells in the tissues. In the abdominal cavity, HFH-11 is expressed in the proliferating cells of the embryonic lung, liver, intestine, cortex of the kidney, and throughout the urinary tract (Fig. 6A and B). Specific HFH-11 hybridization signal was also observed in adipose tissue located dorsal to the vertebrae, in the skin, and in the epithelium of the salivary gland (Fig. 6A and B). These hybridization signals were not observed with the HFH-11 sense probe (data not shown). Enlargement of the embryonic lung depicts expression of HFH-11 in the mesenchyme cells which are fated to differentiate into the endothelium and stroma of the adult lung and in the epithelium of the terminal and proximal bronchioles (Fig. 6C and D). Higher magnification of the embryonic liver displays HFH-11 signals in hepatocytes but not in the blood or hematopoietic cells that reside in the liver at this stage of development (Fig. 6E and F). The embryonic intestine also displays HFH-11 expression in the mesenchyme

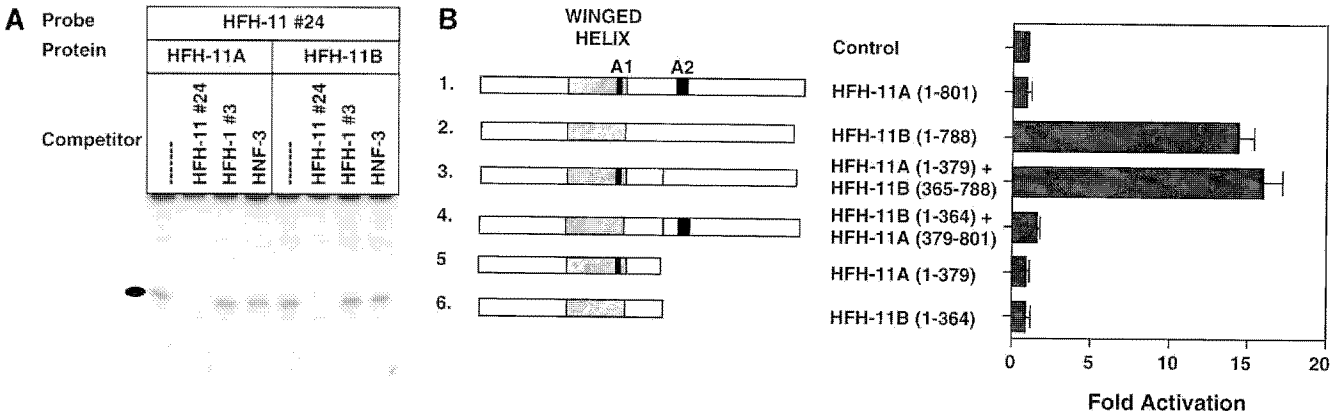


FIG. 4. HFH-11B is a transcriptional activator, while exon A2 sequences inhibit HFH-11A transcriptional activity. (A) EMSA with in vitro-translated HFH-11 protein demonstrate specific protein-DNA complex formation (indicated by the black oval) with the HFH-11 #24 binding site identified by SAAB selection with recombinant HFH-11 protein. Included as competitors are itself and HNF-3 binding sites from in vitro-binding site selection (HFH-1 #3) and from the transthyretin promoter (−111 to −85 bp; HNF-3). (B) Chimeras between HFH-11A and HFH-11B proteins indicate that C-terminal exon A2 sequences inhibit transcriptional activation. By using common *Kpn*I restriction sites, chimeric proteins were created between the HFH-11A and HFH-11B winged helix domain and the C terminus of the other isoform. To analyze for transcriptional activation, wild-type and chimeric CMV HFH-11 expression constructs were cotransfected with the reporter plasmid containing five copies of the HFH-11 24 binding site upstream of a TATA box-driven luciferase gene in Caco-2 cells, and protein extracts were prepared 48 h later and analyzed for luciferase enzymatic activity. The presence of the exon A1 and exon A2 sequences are shown schematically, and the fold activation over empty CMV expression vector is summarized as an average of three separate experiments containing three plates for each cotransfected construct. HFH-11A (construct 1), HFH-11B (construct 2), HFH-11A winged helix and HFH-11B C terminus (construct 3), HFH-11B winged helix and HFH-11A C terminus (construct 4) and C-terminal truncations of HFH-11A (construct 5) and HFH-11B (construct 6) were used. The numbers in parentheses are amino acids.

and in the smooth muscle and epithelial cells (Fig. 6G and H). Enlargement of the cortical region of the embryonic kidney allowed visualization of HFH-11 expression in undifferentiated metanephric mesenchymal cells (4, 19) and in the epithelium of the glomerulus and tubules (Fig. 6I and J). These studies demonstrate that HFH-11 is expressed in proliferating epithelial and mesenchymal cells in the embryonic lung, liver, intestine, and renal cortex.

Winged helix gene expression in epithelial cells of the adult mouse intestinal crypts. In order to determine the expression patterns of winged helix genes in the adult mouse intestine, we performed in situ hybridization studies with antisense rat HNF-3 and rat HFH-11 RNA probes (Fig. 7). Although HFH-11 is expressed in the mesenchymal and epithelial cells of the embryo, its expression pattern in adult intestine is localized to Lieberkühn's crypts found at the base of the villus (Fig. 7A and B). Higher magnification demonstrates that HFH-11 expression is restricted to the proliferating epithelium of Lieberkühn's crypts (53), but it is not expressed in the differentiated Paneth cells located at the base of the crypts (Fig. 7C and D). Thus, HFH-11 possesses a restricted cellular expression pattern in the adult intestinal epithelium that is not exhibited by other intestinal transcription factors (10, 61, 63, 65).

HNF-3α is strongly expressed in the intestinal epithelial cells of Lieberkühn's crypts, but it is also expressed in the differentiated epithelium of the villus (Fig. 7E and F). HNF-3α expression in the differentiated epithelium gradually decreases from the crypt-to-villus axis (Fig. 7E and F), and the pattern of expression is similar to that of the HNF-1 gene (61). Like HFH-11, HNF-3β expression pattern is also restricted to the epithelial cells of the Lieberkühn's crypts in the adult mouse intestine (Fig. 7G and H). These in situ hybridization experiments demonstrate that the winged helix genes possess distinct cellular expression patterns in the epithelium of the adult mouse intestine.

We next identified putative HNF-3 target genes in the intestinal epithelium by using the HNF-3 DNA binding consensus sequence and examined whether they also bound HFH-11. Putative HNF-3 binding sites were found in the mouse homeodomain cdx-2 (37), E-cadherin (22), rat intestinal fatty acid binding protein (Fabpi) (62), human mismatch repair (hMSH2) (76), and human intestinal alkaline phosphatase genes (Fig. 8C). Recombinant HNF-3α (Fig. 8A) and HNF-3β (data not shown) proteins formed a specific protein-DNA complex with HNF-3 binding sites derived from these putative target genes in EMSA. The HNF-1 promoter region is known to bind the HNF-3 isoforms and is transcriptionally activated by HNF-3 expression vectors in cotransfection assays (42). Furthermore, EMSA demonstrated that recombinant HFH-11A (Fig. 8B) and HFH-11B (data not shown) proteins were also able to form specific protein-DNA complexes with sequences from the cdx-2, E-cadherin, and Fabpi genes, but not with the hMSH2 and alkaline phosphatase sites. These experiments identify several potential HNF-3 and HFH-11 target genes which serve important biological functions in the intestinal epithelium.

Liver regeneration markedly induces HFH-11 expression in adult hepatocytes. Our in situ hybridization experiments (Fig. 6E and F) demonstrated that HFH-11 is expressed in proliferating hepatocytes in embryonic liver but its expression is extinguished in the adult liver (Fig. 1C). Because HFH-11 expression is found in human hepatoma HepG2 cells (41), we wanted to examine whether HFH-11 expression could be induced in adult hepatocytes by proliferative signals. Partial hepatectomy induces replication of hepatocytes in the remaining liver lobe, allowing an increase in the number of hepatocytes and regeneration of the liver to its original size. This hepato-

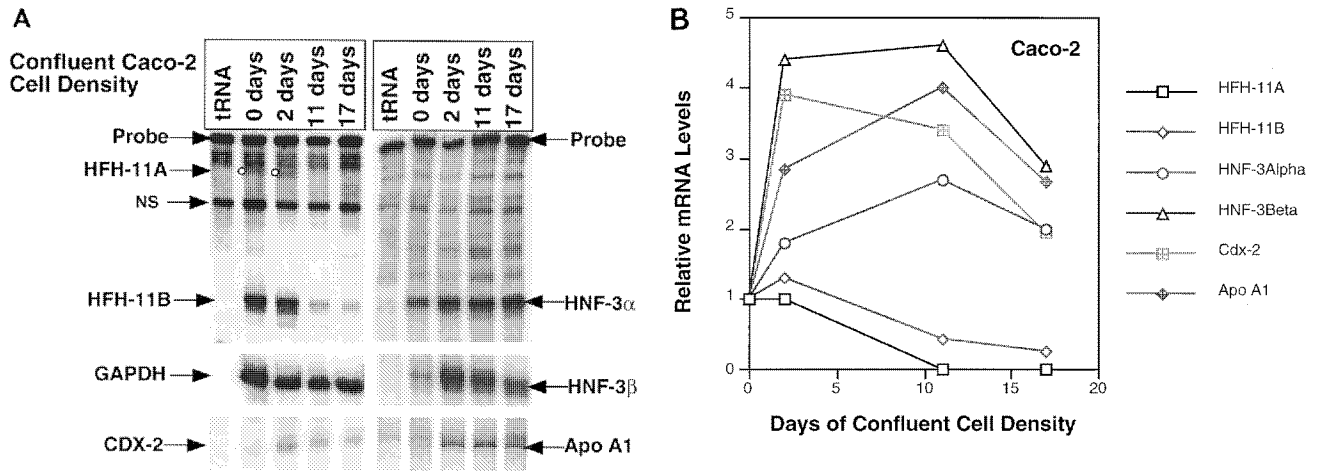


FIG. 5. Expression of HNF-3 and HFH-11 mRNA during intestinal Caco-2 cell differentiation. (A) RNase protection assays with winged helix probes and RNA isolated from Caco-2 cells at different stages of differentiation. Total RNA was isolated from undifferentiated Caco-2 cells (0 days is at 90% confluent cell density, which is 5 days after plating the cells) or differentiated Caco-2 cells (2, 11, and 17 days of confluent cell density, which are 7, 16, and 22 days after plating of the cells, respectively). This RNA was hybridized with antisense RNA probes specific to HFH-11, HNF-3α, HNF-3β, cdx-2, and apolipoprotein A1 (Apo A1), digested with RNase I, and subjected to denaturing polyacrylamide gel electrophoresis. The human HFH-11 RNA probe contained sequences spanning exon A2 (amino acids 366 to 469) which allowed detection of both HFH-11 isoforms, generating RNase-protected fragments of 309 nucleotides for HFH-11A mRNA and 171 nucleotides for HFH-11B mRNA. Hybridizations between RNA probes and tRNA allowed the identification of nonspecific (NS) RNase-resistant digestion products due to secondary structure of the RNA. Indicated on the gels are the positions of the undigested probe and RNase-protected products for HFH-11A, HFH-11B, HNF-3α, HNF-3β, cdx-2, Apo A1, and GAPDH. Note that HFH-11 expression decreases following Caco-2 differentiation (at 11 days), while HNF-3, cdx, and Apo A1 expression increases at the initial stages of Caco-2 cell differentiation (2 days). (B) Graph of relative mRNA expression levels of the winged helix genes during Caco-2 differentiation. RNA expression levels were normalized to the GAPDH control band and plotted as fold increase in mRNA steady-state levels with respect to undifferentiated Caco-2 cells.

cyte replication is driven by the paracrine release of tumor growth factor alpha and hepatocyte growth factor and provides an in vivo model for examining changes in gene expression during cellular proliferation (24). Rat liver RNA was prepared at various times following partial hepatectomy and evaluated for HFH-11 expression levels via RNase protection assays (Fig. 9). After normalization to control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals, a sevenfold increase in HFH-11 expression was observed at 4, 24, and 48 h after partial hepatectomy, which disappeared by 72 h posthepatectomy (Fig. 9). The expression of HFH-11 precedes DNA replication in the proliferating hepatocytes (18 to 28 h posthepatectomy), and its expression continues throughout the mitotic cell cycle (45). In situ hybridization of 24-h regenerating liver with HFH-11 antisense probe demonstrated that its expression was found in most of the hepatocytes (data not shown) which are actively undergoing DNA synthesis at this time following partial hepatectomy (45). No HFH-11 hybridization signals were found in the livers of sham-operated rats (data not shown). These studies demonstrate that HFH-11 expression can be reactivated in replicating adult hepatocytes induced by proliferative signals following partial hepatectomy and liver regeneration.

HFH-11 expression is induced by KGF in the lung and by oxidative stress in endothelial cells. Our embryonic studies demonstrated that HFH-11 was expressed in the mesenchymal cells of the embryonic lung and in the respiratory epithelial cells of the bronchioles. Northern blot analysis demonstrate that low levels of HFH-11 mRNA are expressed in the adult lung and higher levels are expressed in epithelial cell lines derived from lung tumors (Fig. 1B). Previous studies have demonstrated that tracheal administration of KGF caused a transient proliferation of alveolar type II pneumocytes in the rat lung (71). In order to determine whether KGF treatment will induce HFH-11 expression, total rat lung RNA was prepared at various times following intratracheal KGF administration and analyzed for HFH-11 and GAPDH mRNA levels by RNase protection assays (Fig. 10A). These data showed that a threefold increase of HFH-11 mRNA levels was observed in rat lungs at 18 and 24 h following KGF administration and that this increase diminished to untreated levels by the 72-h time point (Fig. 10A). The increase in HFH-11 expression correlates with the proliferation of alveolar type II pneumocytes, which is complete by 72 h after KGF administration (71), but this experiment does not rule out the possibility that KGF directly stimulates HFH-11 expression. Expression of other winged helix family members including HNF-3α, HNF-3β, HFH-4, and HFH-8 was unaffected by KGF treatment (data not shown). As in the case of adult hepatocytes, HFH-11 expression is increased by growth factor stimulation in the lung.

Because the mesenchymal cells in the embryonic lung are fated to differentiate into endothelium, we examined whether HFH-11 expression could be activated in adult endothelial cells in response to oxidative stress. The HMEC line (derived from adult endothelium) was serum starved for 24 h and treated with different concentrations of hydrogen peroxide for 1 h, and then RNA was isolated and analyzed for HFH-11 expression by Northern blot analysis. HFH-11 mRNA was not detectable in untreated HMEC, but its expression was rapidly increased in response to hydrogen peroxide treatment (Fig. 10B). For a control, the same Northern blot was stripped of the HFH-11 probe and hybridized with another winged helix family member (HFH-8) that is expressed in HMEC. In contrast to HFH-11, HFH-8 expression was found in untreated endothelial cells and its expression did not change in response to oxidative stress (Fig. 10B). These data suggest that HFH-11 is expressed in mesenchymal cells of the mouse embryo and its expression can be induced in adult endothelial cells in response to oxidative stress.

HFH-11 expression in the adult testis is restricted to the spermatocytes and spermatids in seminiferous tubules. Northern blot analysis demonstrated abundant HFH-11 expression in the adult testis, which contains a proliferating stem cell

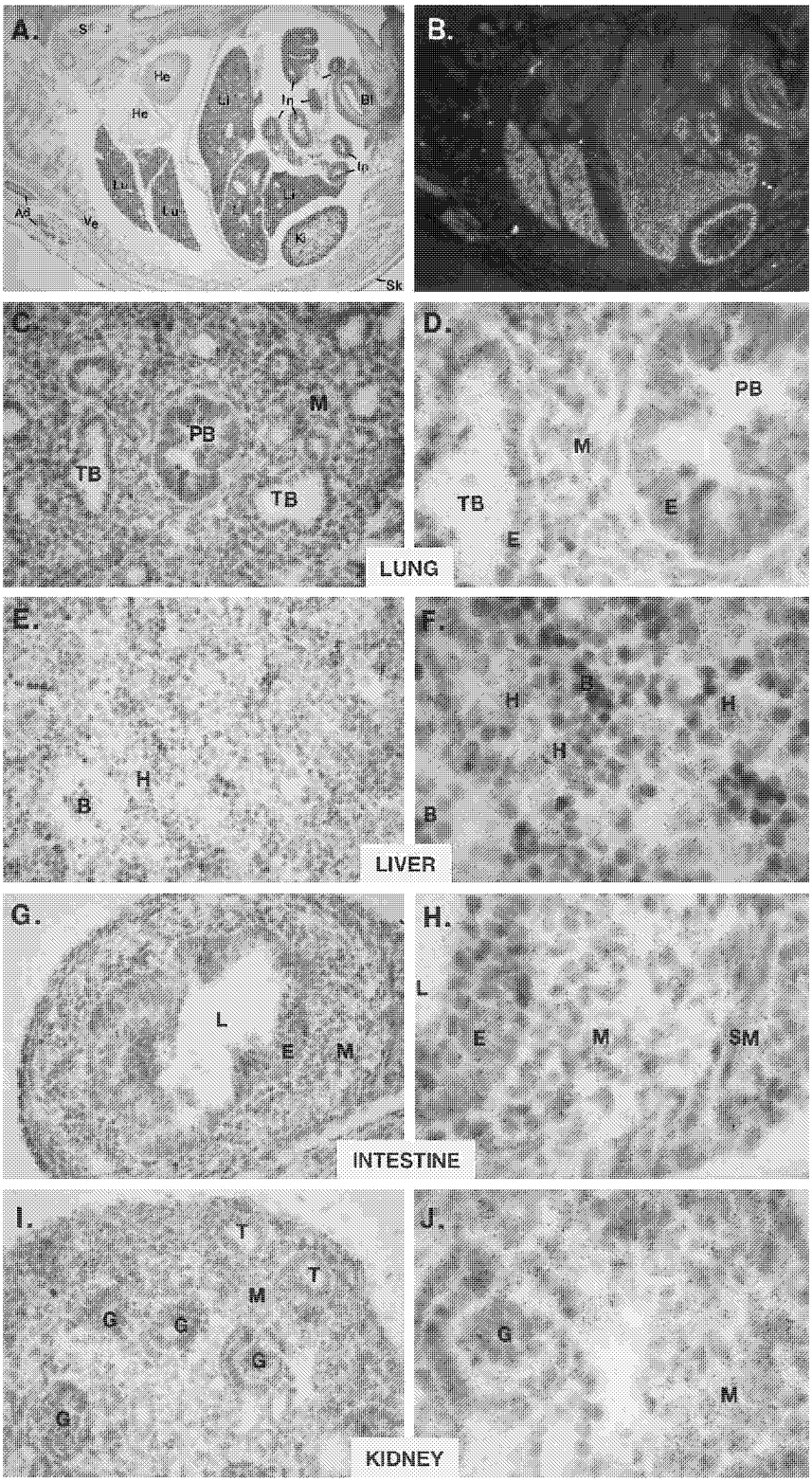


FIG. 6. HFH-11 is expressed in the mesenchymal and epithelial cells of embryonic lung, liver, intestine, and kidney. In situ hybridization of sagittal sections of paraffin-embedded 16 day p.c. mouse embryo with ^{33}P-labeled antisense RNA HFH-11 probe. After hybridization, stringent washes, and autoradiography, dark-field microscopy was used to visualize HFH-11-expressing cells in the tissues. (A) Bright-field photograph of abdominal region of embryo. (B) Dark-field photograph demonstrates that HFH-11 is expressed in embryonic lung (Lu), liver (Li), intestine (In), bladder (Bl), and cortex of the kidney (Ki). HFH-11 hybridization signals are found in adipose tissue (Ad) dorsal to the vertebrae (Ve), skin (Sk), and salivary gland (S) but not in the heart (He). (C and D) Enlargement of embryonic lung shows HFH-11 expression in epithelial cells of the proximal bronchioles (PB) and terminal bronchioles (TB) and in the mesenchyme (M). (E and F) Enlargement of the embryonic liver displays HFH-11 expression in hepatocytes (H) but not in blood (B) or hematopoietic cells. (G and H) Enlargement of embryonic intestine shows HFH-11 in the epithelial (E) cells facing the lumen (L) and in the mesenchymal (M) and smooth muscle (SM) cells. (I and J) Enlargement of the embryonic renal cortex shows HFH-11 expression in the epithelium of the glomerulus (G) and tubules (T) and in the cortical mesenchymal cells.

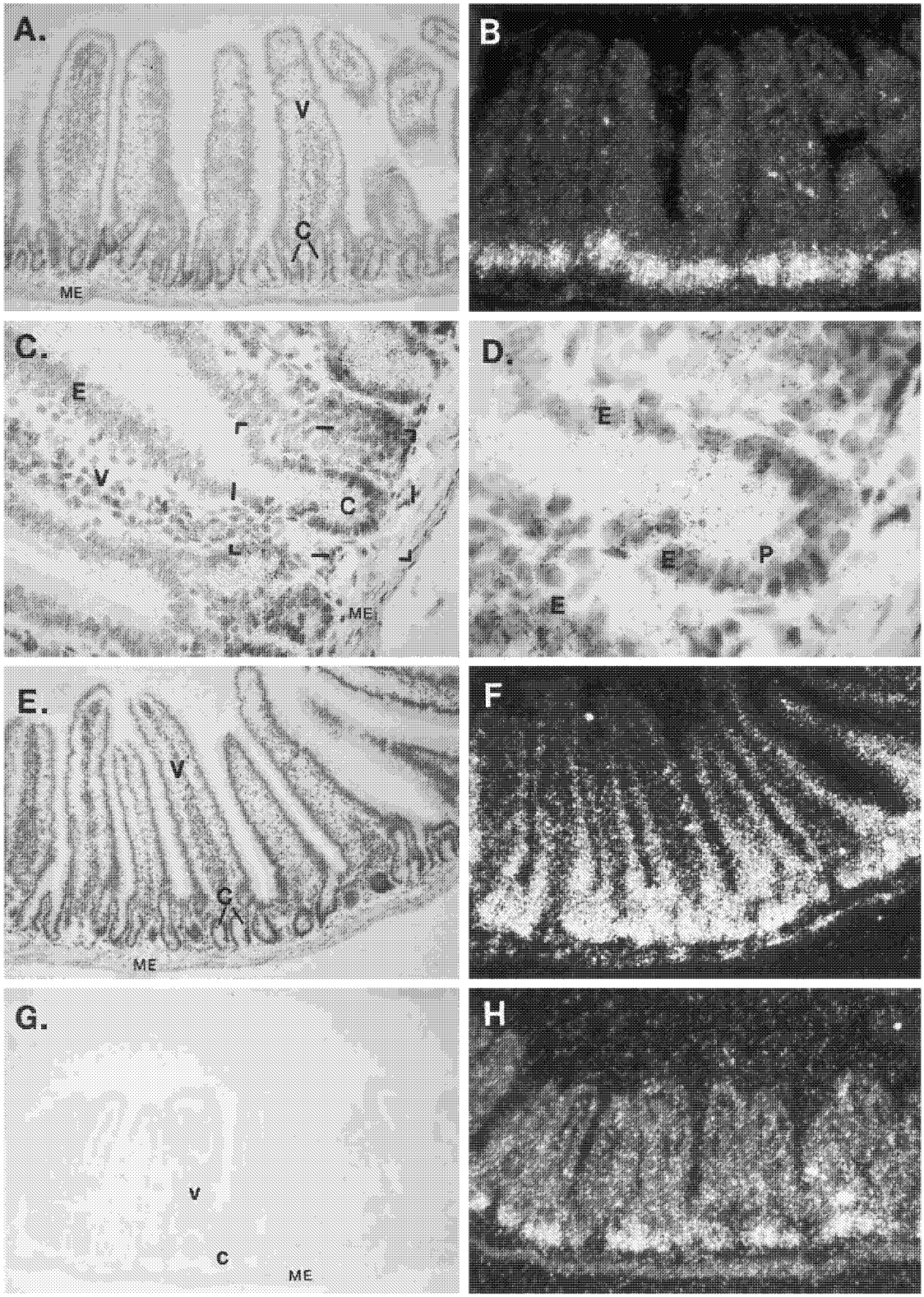
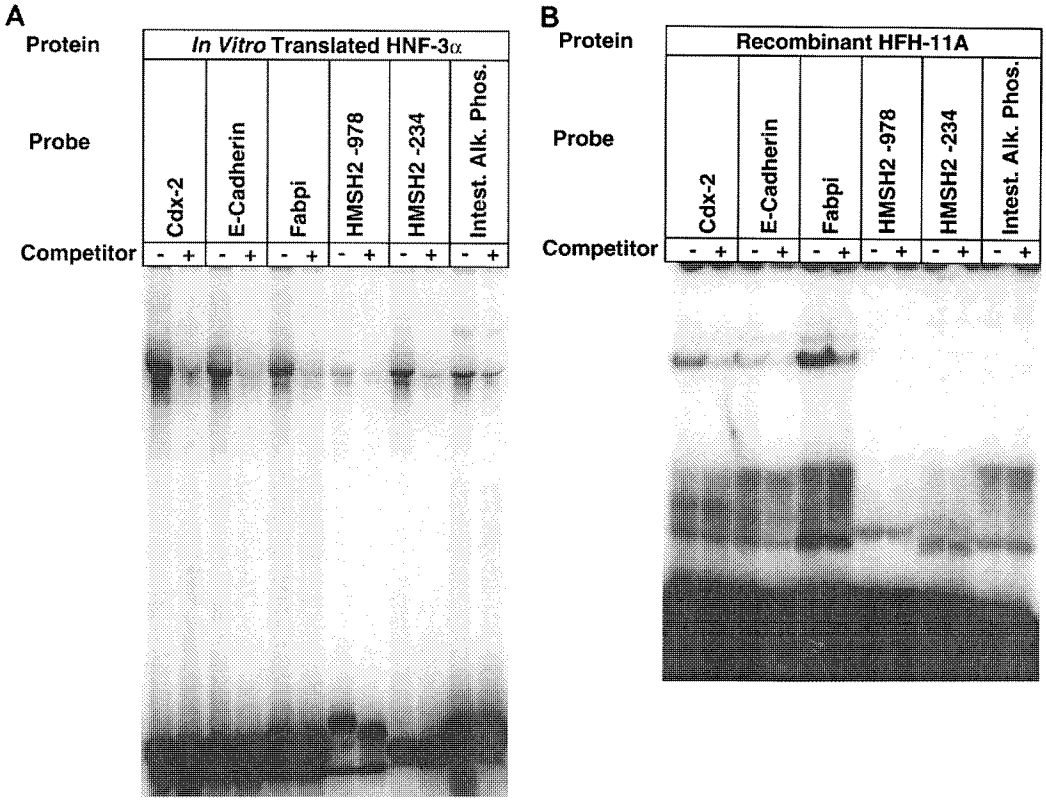


FIG. 7. HFH-11 expression is restricted to the crypt of the adult intestine. Transverse paraffin sections of adult mouse intestine were hybridized with rat ^{33}P-labeled antisense RNA HFH-11 (A to D), HNF-3α (E and F), and HNF-3β (G and H) probes. The dark-field photographs are shown in panels B, F, and H. (A and B) HFH-11 expression is restricted to the epithelium of Lieberkühn's crypts in the adult intestine. Dark-field exposure (B) shows HFH-11 hybridization in Lieberkühn's crypts (C) but not in the intestinal villus (V) or muscularis external region (ME). (C) Higher magnification demonstrates that HFH-11 is restricted to the epithelium (E) of Lieberkühn's crypts but not in the villus. (D) Shown is a higher magnification of the boxed region in panel C, showing HFH-11 hybridization in proliferating epithelium of the crypt but not in the Paneth (P) cells located at the base of the crypts. (E and F) HNF-3α expression is the highest in the epithelium of Lieberkühn's crypts, and its expression in the differentiated epithelium gradually decreases from the crypt-to-villus axis. (G and H) HNF-3β expression is found in the epithelial cells of Lieberkühn's crypts but not the villus.



C

Putative Winged Helix Target Genes in intestinal epithelium:

Gene	Genbank Accession No.	Position	Sequence	HNF-3 Binding	HFH-11 Binding
mouse Cdx-2	U00454	+4716/+4728	TTTGTTTGTTTG	+++	+++
mouse E-Cadherin	X60961	-551/-563	TTTGTTTGTTTT	+++	+
rat Fabpi	M18080	-393/-381	GTTGTTTGTTTT	+++	++
hMSH2	U27346	-986/-974	TTTATTTATTTT	++	-
hMSH2	U27346	-217/-205	TGTGTTTACTCA	++	-
human Alk. Phos.	Y00512	-953/-941	GATGTTTGTTCT	++	-
mouse HNF-1		-17/-29	TCTGTTTACATT	+++	ND
HNF-3 Consensus:			WWTRTTTRYWYD		

FIG. 8. Identification of putative HNF-3 and HFH-11 target genes in intestinal epithelium. HNF-3 consensus sequence was used to search intestinal genes. Putative HNF-3 binding sites were found in the mouse cdx-2 homeodomain transcription factor, rat intestinal fatty acid binding protein (Fabpi), human DNA mismatch repair (hMSH2), and intestinal alkaline phosphatase (Intest. Alk. Phos.) genes. Double-stranded oligonucleotides corresponding to these sequences were used in EMSA with recombinant in vitro HNF-3α (A) or GST–HFH-11A protein (B). Comparable binding activity was obtained with recombinant HNF-3β and HFH-11B proteins, respectively (data not shown). (C) Potential HNF-3 and HFH-11 target gene sequences and summary of DNA binding affinities. Identification of HNF-3 binding site in the HNF-1 promoter was described by Kuo et al. (42). HNF-3 and HFH-11 protein binding affinities were summarized as follows: +++, high; ++, moderate; +, weak; −, none; ND, not done. Indicated is the HNF-3 consensus sequence (50) in which the following nucleotide abbreviations were used: W is A or T, R is G or A, Y is C or T, and D is not C. Note that cdx-2, E-cadherin and fabpi bind both recombinant HNF-3 and HFH-11 proteins.

population that undergo spermatogenesis to replenish the terminal differentiated spermatozoa cells. The seminiferous tubules of the adult testis contain spermatogonia stem cells which undergo proliferation and meiotic division in the spermatocyte stage and form the haploid spermatids which undergo terminal differentiation. We used in situ hybridization to determine HFH-11's cellular expression pattern in the adult mouse testis, which contains seminiferous tubules at different stages of spermatogenesis (Fig. 11). Because HFH-4 expression is restricted to tubules at stages I to VI of spermatogenesis (30), we used it as a control for in situ hybridization comparisons (Fig. 11A). In contrast to HFH-4 expression pattern, HFH-11 is expressed in the spermatocytes and spermatids in the seminiferous tubules throughout the adult male testis. However, HFH-11 is expressed at higher levels in the tubules in stages I to VIII which contain both spermatocytes and spermatid cells (Fig. 11B). Higher magnification shows that HFH-11 was expressed throughout the meiotic spermatocytes and differentiating spermatids of the seminiferous tubules but not in the spermatogonia adjacent to the basement membrane of the seminiferous tubule (Fig. 11C). These studies suggest that HFH-11 expression is found in proliferating epithelial cells of adult tissue that are involved in replenishing a short-lived terminally differentiated cell population.

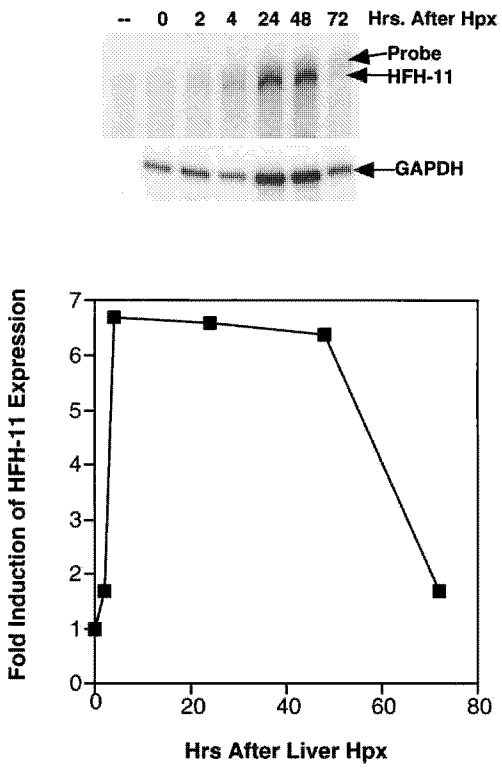


FIG. 9. Hepatocyte proliferation during liver regeneration induces HFH-11 expression. Total RNA from rat livers was isolated at various times (hours) after partial hepatectomy (Hpx) and analyzed for HFH-11 and GAPDH mRNA levels by RNase protection with corresponding rat RNA probes (55). HFH-11 mRNA levels were normalized to GAPDH signals, which we know from a previous study does not change (55), using BioMax one-dimensional image analysis software (Kodak). These data are presented graphically from data generated from one set of regenerating rat livers, and similar results were obtained from a second set of regenerating rat livers by in situ hybridization (data not shown). Note that the rat HFH-11 probe does not distinguish between the HFH-11A and HFH-11B isoforms.

DISCUSSION

The winged helix proteins comprise an extensive family of transcription factors which participate in the differentiation of diverse cellular lineages and function in pattern formation during embryogenesis. In this study, we report on the isolation of two isoforms (HFH-11A and HFH-11B) of the winged helix transcription factor family from human colon carcinoma HT-29 cells which arise via differential splicing of the same gene. Similar to other transcription factors (67), these isoforms exhibit different transcriptional activities, but only the HFH-11B isoform is transcriptionally active in cotransfection assays. RNase protection assays show that the transcriptionally active HFH-11B isoform is the most prominent isoform in undifferentiated Caco-2 and HT-29 cells (Fig. 5 and data not shown), and Western blot analysis confirms expression of HFH-11 protein in these cell lines. We demonstrated that Caco-2 differentiation toward the intestinal enterocyte lineage resulted in decreased expression of HFH-11 and reciprocal increases in HNF-3 expression. In situ hybridization experiments of 16 day p.c. mouse embryos demonstrate that HFH-11 is expressed in proliferating mesenchymal and epithelial cells of the embryonic lung, liver, intestine, and renal cortex. In spite of this broad expression pattern in the mouse embryo, the expression pattern of HFH-11 in the adult intestine is restricted to the epithelial cells of Lieberkühn's crypts. HFH-11 exhibits low mRNA levels in adult liver and lung organs, but its expression is reactivated in hepatocytes in response to proliferative signals after partial hepatectomy, in lungs after KGF administration, and in adult endothelial cells in response to oxidative stress. These studies suggest that the broad expression pattern in proliferating mesenchymal and epithelial cells in the embryo becomes restricted to proliferative cells in adult tissues and that its expression is reactivated in adult cells via proliferative signals or in response to oxidative stress.

HFH-11 expression is also observed in a number of epithelial cell lines derived from tumors, suggesting that it may play a role in cellular proliferation and transformation. The C-terminal region of the HFH-11 protein contains several putative MAP kinase phosphorylation sites (20, 48, 68), a feature that allows activation of transcriptional activity in response to growth control signalling (39). In support of the authenticity of these phosphorylation sites, the C terminus of the HFH-11 protein (HFH-11B amino acids 528 to 788) has been previously isolated by screening of expression libraries with M-phase kinase followed by detection with the MPM2 monoclonal antibody (74), which recognizes phosphoamino acid-containing epitopes on many M-phase proteins. Using chimeric constructs of the HFH-11 isoforms, we show that the transcriptional activation function resides in the C-terminal sequences and that insertion of exon A2 sequences in this C-terminal region represses this activity. The HFH-11A protein also contained a 15-amino-acid insertion (exon A1) at the C terminus of the winged helix domain located adjacent to the basic amino acids of the wing 2 region which are involved in base-specific contact of the minor groove (12). DNA binding experiments indicated that the exon A1 amino acid insertion does not alter the DNA binding specificity of the HFH-11A winged helix domain from that of the HFH-11B protein (data not shown). However, putative protein phosphorylation sites for the meiosis-activated myelin basic protein kinase (p44mpk) and MAP2 kinase (13, 68) are found in the exon A1 sequences. Mitogen-activated phosphorylation of the DNA binding domain has been observed in a number of transcription factors to transiently inactivate their DNA binding activity during the cell cycle (9, 44). Phosphorylation of the exon A1 protein sequence may therefore confer a potential mechanism by which to inhibit DNA recognition by the HFH-11A protein and prevent its inhibitory effect on transcriptional activation. Furthermore, HFH-11 protein contains two PEST sequences which are known to mediate rapid protein degradation in immediate-early-response AP-1 transcription factors (56). The HFH-11 proteins therefore possess features in common with transcription factors that respond to proliferative signalling.

Our differentiation studies of Caco-2 cells suggest that this human colon carcinoma cell line begins to differentiate toward the enterocyte lineage at 2 days of confluent cell density with strong induction of HNF-3β and cdx-2 expression which coincides with the increase in expression of an HNF-3 target gene, apoliprotein A1 (31). Interestingly, induction of HNF-3β and cdx-2 expression correlates with an increase in the cyclin-dependent kinase inhibitor, p21 (WAF1/CIP1) (27), which is expressed during terminal differentiation of a variety of cell lineages (52). However, HFH-11 is still expressed in Caco-2 cells that are beginning to differentiate (2 days), suggesting that HFH-11 may regulate genes mediating the transition between proliferating intestinal epithelial cells and enterocyte differentiation which occurs after exiting the cell cycle (53). We show that HFH-11 expression begins to decline by 5 days of continuous growth at high cell density and its expression is significantly reduced in fully differentiated Caco-2 cells, which express an extensive array of enterocyte-specific marker genes

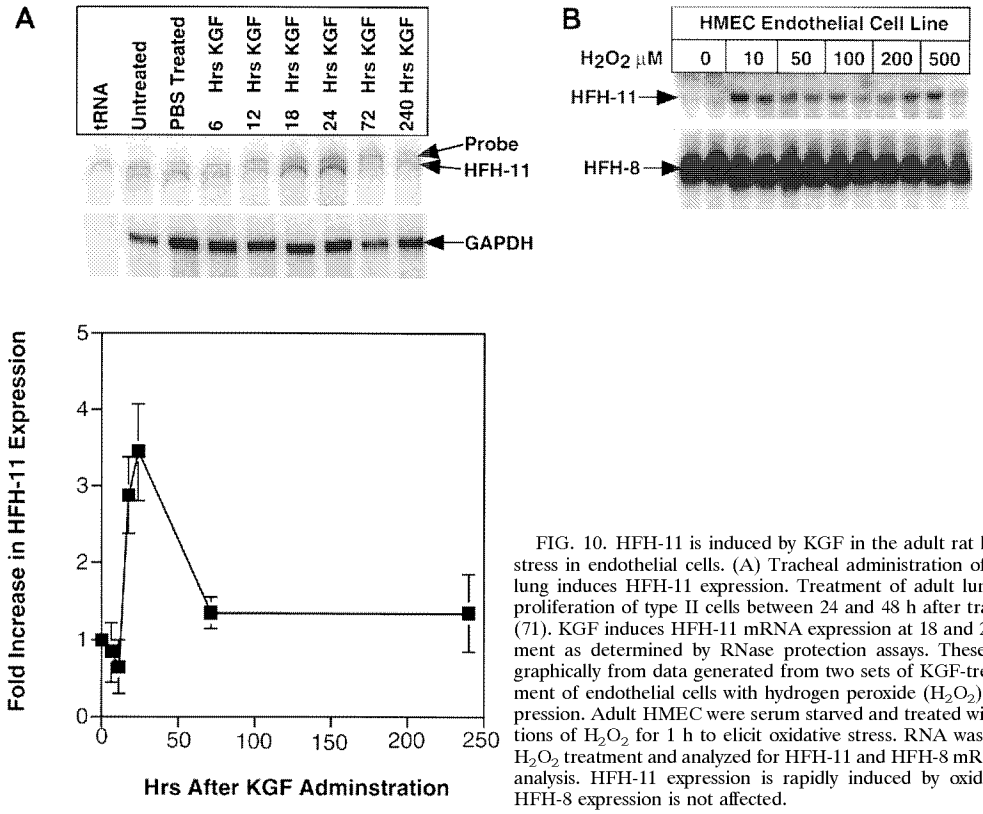


FIG. 10. HFH-11 is induced by KGF in the adult rat lung and by oxidative stress in endothelial cells. (A) Tracheal administration of KGF into the adult lung induces HFH-11 expression. Treatment of adult lungs with KGF causes proliferation of type II cells between 24 and 48 h after tracheal administration (71). KGF induces HFH-11 mRNA expression at 18 and 24 h after KGF treatment as determined by RNase protection assays. These data are presented graphically from data generated from two sets of KGF-treated rats. (B) Treatment of endothelial cells with hydrogen peroxide (H_2O_2) induces HFH-11 expression. Adult HMEC were serum starved and treated with various concentrations of H_2O_2 for 1 h to elicit oxidative stress. RNA was isolated after 1 h of H_2O_2 treatment and analyzed for HFH-11 and HFH-8 mRNA via Northern blot analysis. HFH-11 expression is rapidly induced by oxidative stress, whereas HFH-8 expression is not affected.

(11). HNF-3 and *cdx-2* gene expression continues in fully differentiated Caco-2 cells, suggesting that both play an important role in the maintenance of the differentiated state. In support of this hypothesis, ectopic expression studies of the *cdx-2* gene in undifferentiated intestinal IEC-6 cells caused exiting from the cell cycle and a concomitant terminal differentiation (64).

Although HNF-3α and HNF-3β exhibit similar expression patterns in embryonic gut endoderm (47), our in situ hybridization data demonstrates that their expression pattern diverges in the adult intestine. HNF-3β expression is restricted to the epithelial cells of Lieberkühn's crypts and exhibits an expression pattern similar to that of HFH-11. The fact that HNF-3β levels are markedly higher in the initial stages of Caco-2 cell differentiation (Fig. 5A) suggests that HNF-3β's expression may be restricted to postmitotic epithelial cells beginning their differentiation program (16). By contrast, HNF-3α demonstrates strong expression in the intestinal crypt cells, and its expression in the differentiated epithelium gradually decreases from the crypt-to-villus axis. Interestingly, a number of our potential HNF-3 and HFH-11 target genes exhibit an epithelial cell expression pattern that decreases from the crypt-to-villus axes. These genes include the fabpi gene, which is expressed in postmitotic epithelial cells of the crypts (16) and the HNF-1 (42, 61) and mouse homeodomain cdx-2 (37) transcription factor genes whose products regulate genes important for intestinal epithelial function (63, 64, 77). The high levels of expression of HNF-3 and HFH-11 in the epithelial cells of the crypts may play a role in establishing the expression pattern of these putative target genes. We also show that the human mismatch repair gene (hMSH2) may be a putative target gene of HNF-3β (Fig. 8), which exhibits a parallel restricted expression pattern (76). The hMSH2 protein is critical for fidelity of DNA replication in gut epithelium, since its gene is mutated in a number of human colon carcinomas (25). E-cadherin is another potential winged helix protein target gene in the intestinal epithelium, and its appropriate expression pattern is critical for epithelial cell migration and viability (33). Our studies identify unique expression patterns of the winged helix protein in the epithelium of the adult intestine and potential target genes necessary for organ function.

In situ hybridization studies of adult testis demonstrated that HFH-11 is expressed in spermatocytes and spermatids of the seminiferous tubules undergoing spermatogenesis. As in the adult intestine, HFH-11 expression is found in proliferating epithelial cells involved in replenishing a short-lived differentiated cell type. HFH-11 expression was found in all of the seminiferous tubules but exhibited stronger hybridization signals in seminiferous tubules at differentiation stages I to VIII. HFH-11 is abundantly expressed in meiotic spermatocytes and differentiating spermatids, but not in the spermatogonia stem cell, a single cell layer located adjacent to the basement membrane. The HFH-11 expression pattern also overlaps with that of the winged helix family member HFH-4, which is found in seminiferous tubules at stages I to VII (30). The winged helix family exhibits a distinct expression pattern in the adult testis and may play important roles in spermatogenesis and terminal differentiation of spermatids to spermatozoa.

Hepatocyte proliferation in the embryonic liver is critical for the formation and organization of the adult liver (78). Our studies demonstrate that HFH-11 is expressed in proliferating hepatocytes of the embryonic liver, but its expression is extinguished in adult liver. Hepatocyte proliferation in response to partial hepatectomy reactivated HFH-11 expression between 4 and 48 h after partial hepatectomy which precedes the initia-

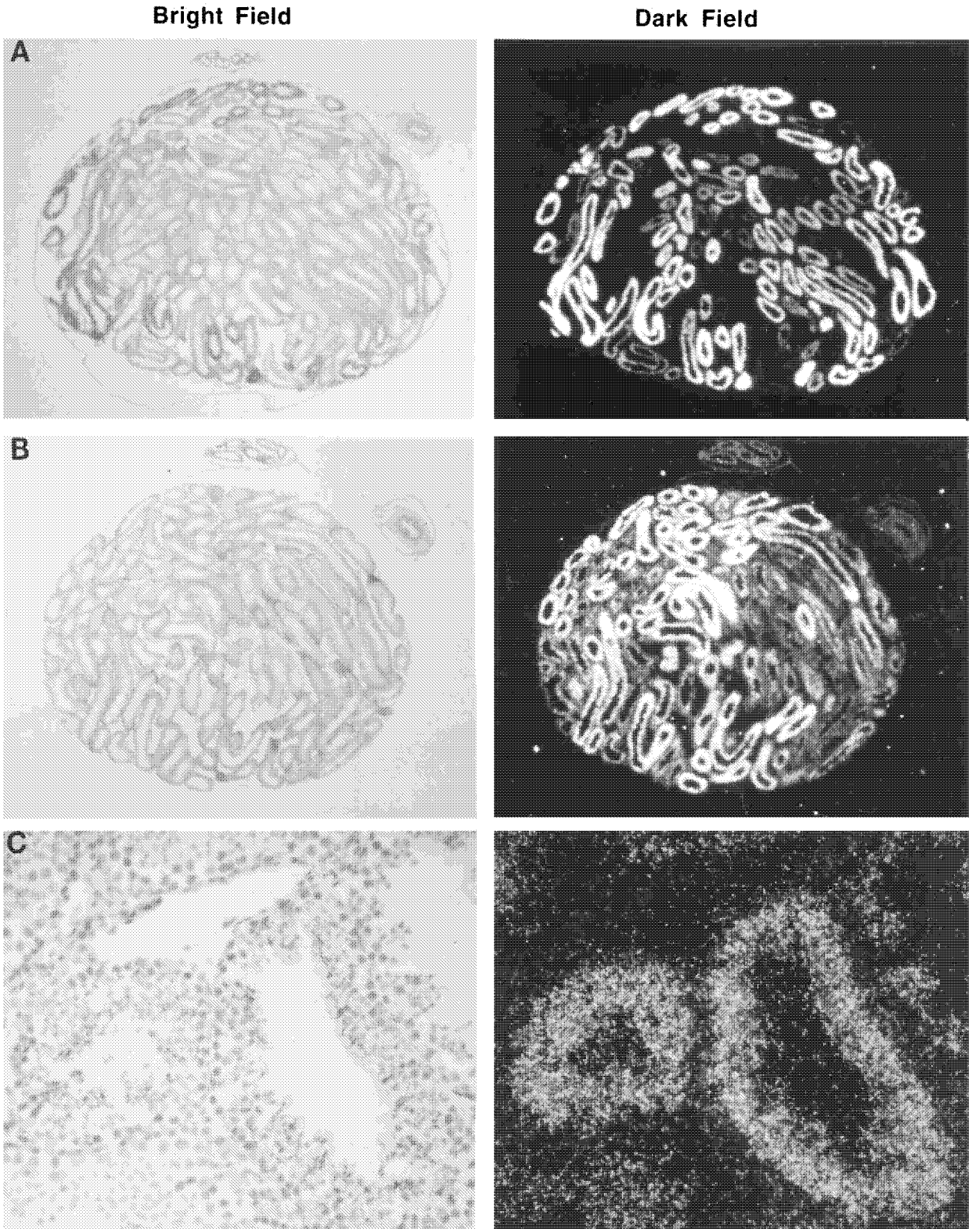


FIG. 11. HFH-11 is expressed during spermatogenesis in adult mouse testis. In situ hybridization of paraffin-embedded mouse adult testis with HFH-4 (A) and HFH-11 (B) antisense RNA probes. (A) HFH-4 hybridization is found in seminiferous tubules at stages I to VI of spermatogenesis (30). (B) HFH-11 expression is found in all seminiferous tubules but is expressed at higher levels in stages I to VIII containing both spermatocytes and spermatids. (C) Higher magnification of seminiferous tubules displaying HFH-11 expression in spermatocytes and spermatids (located toward the lumen of the seminiferous tubule). HFH-11 is not expressed in spermatogonia stem cells located adjacent to basement membrane of seminiferous tubules. No hybridization signals were found in the testis when the HFH-11 sense probe was used (data not shown).

tion of DNA replication (45) and coincides with the decline of immediate-early transcription factor protein levels (66). The kinetics of HFH-11 activation during liver regeneration suggests that it may be a delayed early transcription factor. This hepatocyte replication is driven by the paracrine release of tumor growth factor alpha and hepatocyte growth factor (24), suggesting that HFH-11 expression is also regulated by these growth factors. However, hepatocytes remain differentiated during this stage of proliferation, as evidenced by the expression of many of the liver-enriched transcription factors and hepatocyte-specific genes not influenced during liver regeneration (26, 55, 66). The induction of the HFH-11 protein throughout the mitotic cycle of the proliferating hepatocyte allows us to speculate that HFH-11 plays a role in maintaining the hepatocyte phenotype during this cellular replication as well. HFH-11 expression is compatible with differentiation in that many of the liver-enriched transcription factors and target genes are already expressed in 16 day p.c. embryonic livers (81). Furthermore, the HFH-11B isoform is expressed in human hepatoma HepG2 cells, which continues to express hepatocyte-specific genes and transcription factors (21, 41, 55). The expression pattern of HFH-11 in embryonic and regenerating liver supports the hypothesis that HFH-11 plays a role in the maintenance of a differentiated phenotype during hepatocyte proliferation.

Our studies show that HFH-11 is expressed in the mesenchymal and epithelial cells of the embryonic lung and is reactivated in these adult cell types in response to injury and repair. Previous studies showed that tracheal injection of KGF in the adult rat lung elicits a transient proliferation of alveolar type II pneumocytes in the alveolar sac (71). We show that HFH-11 expression was induced between 18 and 24 h after KGF administration and returned to normal after 72 h, which coincided with the completion of cellular proliferation (71). We show that HFH-11 mRNA is absent in mesenchyme-derived endothelial cell lines, but its expression is reactivated in endothelial cells in response to oxidative stress induced by H_2O_2 treatment. Treatment of different cell lines with H_2O_2 rapidly activates the MAP kinase pathway and corresponding transcriptional induction of its downstream c-Jun and c-Fos genes (29). Furthermore, H_2O_2 selectively stimulated Big MAP kinase 1 enzymatic activity in a wide variety of cell lines (1). Activation of these protein kinase signalling pathways constitutes possible mechanisms by which HFH-11 expression is induced in response to oxidative stress.

We also show that HFH-11 is expressed in the embryonic renal cortex which contains mesenchymal cells that differentiate into epithelial cells of the renal nephrons. Consistent with findings in the other organs, preliminary in situ hybridization studies indicate that only a few cells throughout the renal cortex continue to express HFH-11 in the adult kidney (data not shown). HFH-11 expression may be induced throughout the renal cortex in response to renal damage or injury. HFH-11 may also play a role in other epithelial and mesenchymal differentiation in the embryo, as we observed HFH-11 expression in the urinary system, skin, nasal cavity, tongue, and in the choroid plexus in the ventricles of the brain (data not shown).

In summary, although the embryonic expression pattern of HFH-11 is somewhat broad, HFH-11 utilization is restricted to adult organs containing proliferating cells involved in replenishing differentiated cell populations or in response to growth factors released during injury and repair.

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